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Original article

Expression of $CysLT_2$ receptors in asthma lung, and their possible role in bronchoconstriction

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CysLT	cysteinyl leukotriene
ICS	inhaled corticosteroids
LABA	long-acting β_2 receptor agonist

ABSTRACT

Background: The expression and functional role of $CysLT_2$ receptors in asthma have not been clarified. In this study, we evaluated $CysLT_2$ receptors expression, and effects of $CysLT_2$-and $CysLT_{1/2}$-receptor antagonists on antigen-induced bronchoconstriction using isolated lung tissues from both asthma and non-asthma subjects.

Methods: $CysLT_1$ and $CysLT_2$ receptors expression in asthma and non-asthma lung tissue preparations was examined in immunohistochemistry experiments, and their functional roles in antigen-induced bronchoconstriction were assessed using ONO-6950, a dual $CysLT_{1/2}$-receptor antagonist, montelukast, a $CysLT_1$ receptor antagonist, and $BayCysLT_2RA$, a $CysLT_2$ receptor-specific antagonist.

Results: $CysLT_1$ receptors were expressed on the bronchial smooth muscle and epithelium, and on alveolar leukocytes in 5 in 5 non-asthma subjects and 2 in 2 asthma subjects. On the other hand, although degrees of $CysLT_2$ receptors expression were variable among the 5 non-asthma subjects, the expression in the asthma lung was detected on bronchial smooth muscle, epithelium and alveolar leukocytes in 2 in 2 asthma subjects. In the non-asthma specimens, antagonism of $CysLT_2$ receptors did not affect antigen-induced bronchial contractions, even after pretreatment with the $CysLT_1$-receptor specific antagonist, montelukast. However, in the bronchus isolated from one of the 2 asthma subjects, antagonism of $CysLT_2$ receptors suppressed contractions, and dual antagonism of $CysLT_1$ and $CysLT_2$ receptors resulted in additive inhibitory effect on anaphylactic contractions.

Conclusions: $CysLT_2$ receptors were expressed in lung specimens isolated from asthma subjects. Activation of $CysLT_2$ receptors may contribute to antigen-induced bronchoconstriction in certain asthma population.

Introduction

Cysteinyl leukotrienes (CysLTs), LTC_4, LTD_4 and LTE_4, are inflammatory mediators derived from the 5-lipoxygenase pathway of arachidonic acid metabolism.[1] As these mediators are known to be deeply involved in bronchial asthma via activation of the $CysLT_1$ receptor,[2–4] selective antagonists of this receptor, including pranlukast,[5,6] montelukast,[7] and zafirlukast[8] have widely been used as therapeutic agents for bronchial asthma. On the other hand, a number of studies have cloned and characterized another receptor subtype of CysLTs termed $CysLT_2$ receptor.[9–11] This receptor, like the $CysLT_1$ receptor,[12] is a G-protein-coupled receptor with an amino acid sequence 38% identical to that of the $CysLT_1$ receptor.[9] $CysLT_2$ receptor mRNA has been detected in a number of human organ and tissues, including lung macrophages, airway smooth muscles, and peripheral blood leukocytes,[9] and its expression has been identified on the mucus gland and nasal mucosal epithelium of patients with chronic rhinosinusitis, or allergic nasal vascular smooth muscles.[13,14] As for endogenous

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ligands binding, it is reported that CysLTs order of binding to human $CysLT_1$ receptors is $LTD_4 > LTC_4 = LTE_4$, while that for $CysLT_2$ receptors is $LTC_4 = LTD_4 > LTE_4$,[10] Based on these findings, it is expected that the $CysLT_2$ receptor is involved in the pathophysiology of bronchial asthma. However, it is unclear where $CysLT_2$ receptors are histologically expressed in airway tissues of asthma subjects.

It is well known that CysLTs exert potent contractile action on human bronchial smooth muscles through activation of $CysLT_1$ receptors. Indeed, *in vitro* antigen-induced contraction of passively sensitized human bronchial tissue is markedly suppressed by a pretreatment with $CysLT_1$ receptor antagonists.[4,15,16] However, although $CysLT_2$ receptors are also expressed in bronchial smooth muscles,[9] their functional role in bronchial contraction has not been clarified. In this study, we evaluated CysLT receptors expression and effects of ONO-6950, dual $CysLT_{1/2}$ antagonists and Bay-$CysLT_2$RA,[17] a $CysLT_2$ receptor-specific antagonist on antigen-induced bronchoconstriction using isolated lung tissues from both asthma and non-asthma subjects.

Methods

Subjects

Macroscopic normal portions of lung tissue were obtained from 21 non-asthma and 2 asthma subjects (Table 1) during lung cancer surgery at Ogaki Municipal Hospital (Ogaki, Japan). All subjects provided their informed consents for use of the collected materials in medical research under a protocol approved by the Ethical Committees of Ogaki Municipal Hospital and Kyoto Pharmaceutical University (Kyoto, Japan). Both asthma subjects were diagnosed with bronchial asthma at Ogaki Municipal Hospital in 2003. As shown in Table 1, asthma subject 1 and 2 were treated with inhaled corticosteroid (ICS)/long-acting β_2 receptor agonist (LABA) (fluticasone/salmeterol, Adoair®) at medium (250 μg, twice a day) and low (250 μg, once a day) doses, respectively. In addition, the asthma subject 1 had been treated with montelukast (Singulair®) for approximately 3 months from a day of approximately 1 year before the surgery.

Table 1
Background of lung tissue donors.

	Non-asthma subjects	Asthma subject 1	Asthma subject 2
Age, yr	66.1 ± 12.5	62	60
Age at asthma onset (yr)		54	52
Gender	18 male/3 female	Female	Female
ICS/LABA (Fluticasone/salmeterol, Adoair®)		Yes (medium dose)	Yes (low dose)
FEV_1 (% predicted)		90.3	87.4
Peripheral blood leukocytes (cells/μl)		8440	3920
Neutrophils (%)		39.7	49.7
Eosinophils (%)		6.8	1.0
Basophils (%)		1.2	1.3
Monocytes (%)		5.1	4.1
Lymphocytes (%)		47.2	43.9
Platelets (cells/μl)		319000	201000
Total serum IgE (IU/ml)		838	Not detected
Smoker		No	Yes

In asthma subject 1, Adoair® therapy (250 μg, twice a day) had been continued until the lung cancer surgery, and the lung function and hematological data were collected approximately 6 months before and 1 week after the surgery, respectively. In asthma subject 2, Adoair® therapy (250 μg, once a day) had been continued until 2 years after the surgery when the lung function and hematological data were collected.

How the collected lung samples were used for various experiments is summarized in our online supplementary material (Supplementary Table 1).

Histological and immunohistochemical studies

The lung tissues were fixed in 10% formaldehyde, embedded in paraffin wax, and cut into 4 (4-μm thick) serial sections. Two of these sections were stained first with hematoxylin and eosin (HE), and then with alcian blue/periodic acid-Schiff (AB/PAS). The other 2 sections were used in immunohistochemistry for detection of $CysLT_1$ and $CysLT_2$ receptors expression. After antigenicity activation, the sections were treated with 3% hydrogen peroxide solution, washed with 50 mM Tris–HCl buffer (pH 7.4), and blocked with 1% bovine serum albumin. The sections were then stained with rabbit polyclonal anti-human $CysLT_1$ receptor antibody (LS-A1317, LifeSpan Biosciences, Seattle, WA, USA at a dilution of 1:600) or with rabbit polyclonal anti-human $CysLT_2$ receptor antibody (120560, Cayman Chemical, Ann Arbor, MI, USA at a dilution of 1:300) overnight at room temperature. After washing with 50 mM Tris–HCl buffer (pH 7.4), the sections were treated with anti-rabbit biotinylated secondary antibody for 30 min at 4 °C, washed with 50 mM Tris–HCl buffer (pH 7.4) once again, and stained with streptavidin-horseradish peroxidase for 30 min. Coloring was then developed by soaking the sections in 3,3′-diaminobenzidine solution for 10 min, followed by counterstaining with a hematoxylin solution.

CysLT receptor antagonists

Montelukast, a $CysLT_1$ receptor antagonist, Bay$CysLT_2$RA,[17] a $CysLT_2$ receptor antagonist, and ONO-6950, a dual $CysLT_{1/2}$ receptor antagonist, were synthesized in Ono Pharmaceutical Co., Ltd. (Osaka, Japan) and used in this study as CysLT receptor antagonists.

The chemical formula of ONO-6950 is 4,4'-[4-fluoro-7-(2-{4-[4-(3-fluoro-2-methylphenyl)butoxy]phenyl}ethynyl)-2-methyl-1*H*-indole-1,3-diyl]dibutanoic acid.

LTD_4-induced intracellular calcium mobilization

Human $CysLT_1$ ($hCysLT_1$) receptor- and human $CysLT_2$ ($hCysLT_2$) receptor-expressing cells were generated by transfecting each expression vector into Chinese hamster ovary (CHO)-K1 cells as described in Supplementary Methods.

Calcium mobilization assays were carried out using Fura-2 AM-loaded CHO-K1 cells stably expressing $hCysLT_1$ or $hCysLT_2$ receptor. The cells were cultured in Ham's F-12 medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum and 0.5 mg/ml geneticin (Invitrogen) in 5% CO_2 at 37 °C. The cells were seeded into 96-well special optics flat clear bottom black polystyrene TC-treated microplates (Corning, Corning, NY, USA) at 3×10^4 cells/well and then incubated in 5% CO_2 for 24 h at 37 °C. The cells were treated with 5 μM Fura-2 AM (Dojindo, Kumamoto, Japan) in Hanks' Balanced Salt Solution (HBSS) containing 20 mM HEPES and 2.5 mM probenecid (Sigma–Aldrich, St. Louis, MO USA) for 1 h at 37 °C. After washing with HBSS containing 20 mM HEPES, various concentrations of montelukast, Bay$CysLT_2$RA, ONO-6950 or vehicle (0.1% DMSO) were added to the cells, and the cells were incubated, protected from light, for another 30 min at room temperature.

LTD_4 (100 nM for $hCysLT_1$ and 0.3 nM for $hCysLT_2$ receptor) was next added to the cells, and intracellular calcium mobilization was determined by the ratio of fluorescence intensity (f340/f380) measured at 500 nm every 3 s for 4.5 min using a Functional Drug Screening System (FDSS 3000, Hamamatsu Photonics, Hamamatsu, Japan).

Antigen- or LTD_4-induced contraction in isolated human bronchial strips

As described in our previous report,[15] bronchi (outer diameter 2–6 mm) were isolated from macroscopic normal portions of the lung tissue and cut into 1.5 mm wide spirals. In the experiments for evaluation of the effects of CysLT antagonists on antigen-induced contraction, the bronchi were passively sensitized by incubation in human atopic serum (RAST score = 4 <) for 2 h at 37 °C. Bronchial strips (2 cm long, outer diameter 2–4 mm) were then cut and suspended under an isotonic resting tension of 300 mg at 37 °C in a Magnus bath containing Tyrode's solution gassed with 95% O_2–5% CO_2. Before starting the experiments, acetylcholine (5 μM) and then histamine (10 μM) were repeatedly applied to the preparations until almost equal contractions were obtained.

When the resting tonus of the histamine-treated washed smooth muscle had stabilized, guinea pig serum albumin (Sigma–Aldrich) at final concentration of 0.1 or 1 mg/ml was added to prevent adsorption of ONO-6950 and montelukast to the inner wall of the Magnus bath. Just after addition of guinea pig serum albumin, ONO-6950 (100, 300 or 1000 nM), Bay$CysLT_2$RA (100 or 300 nM), montelukast (30 or 100 nM), or DMSO (final concentration: 0.1%) was applied. Antigen (mite extract from *Dermatophagoides farinae*: 3 μg/ml) or LTD_4 (30 nM) challenge was initiated 30–35 min after treatment with each CysLT antagonist. As previously reported,[2,18] a cyclooxygenase inhibitor, indomethacin (Sigma–Aldrich, 3 μM) and an anti-histamine drug, pyrilamine (Sigma–Aldrich, 1 μM) were applied 10 and 5 min before antigen challenge, respectively, to make antigen-induced contractile response dependent on CysLTs activity. Data, expressed as % of 10 μM histamine-induced contraction, were then calculated using height 30 min before the challenge as baseline.

In experiments using LTD_4 as a smooth muscle constrictor, passive sensitization of bronchial tissue was not performed, and neither indomethacin nor pyrilamine was used.

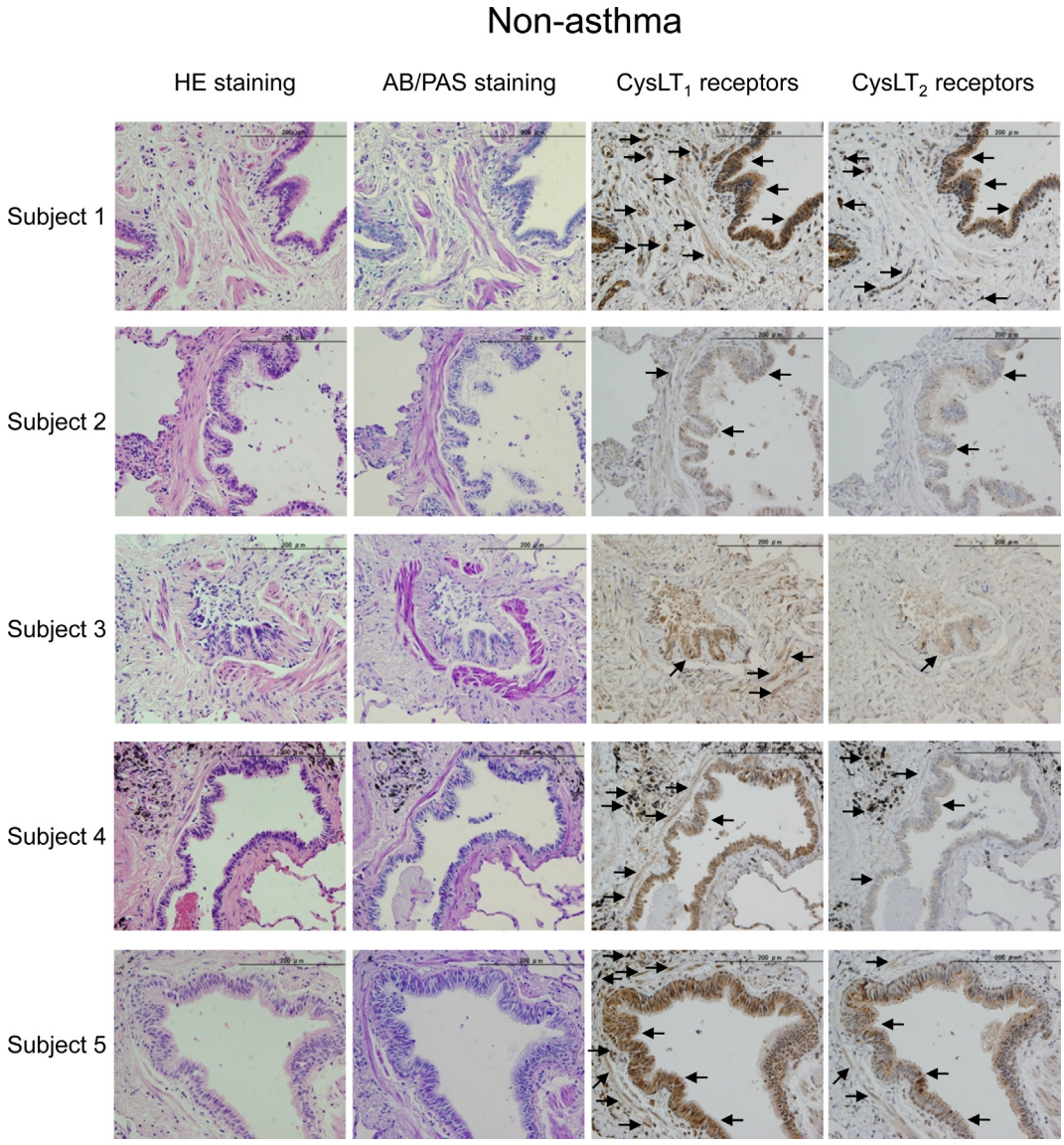

Fig. 1. Immunohistochemical micrographs of lung tissues from the 5 non-asthma subjects. The tissues were cut into 4 serial sections (4-μm thick). Two of the sections were stained with hematoxylin and eosin (HE), and then with alcian blue/periodic acid-Schiff (AB/PAS). The other two sections were used in immunohistochemical determination of $CysLT_1$ and $CysLT_2$ receptors expression. Arrows show representative immunoreactive sites for each receptor.

Results

Immunohistochemical detection of $CysLT_1$ and $CysLT_2$ receptors

As shown in Figure 1, the specimens isolated from all 5 non-asthma subjects revealed $CysLT_1$-receptor immunoreactivity in the bronchial epithelium, bronchial smooth muscle layer, and alveolar leukocytes, although the degree of this receptor's expression was relatively low in subject 2. On the other hand, $CysLT_2$ receptors were expressed predominantly on the bronchial epithelium of non-asthma subjects. $CysLT_2$ receptors expression on bronchial smooth muscle was observed in non-asthma subject 4 and 5, and that on alveolar leukocytes was in non-asthma subject 1, 4 and 5 (Fig. 1).

Like the specimens from the 5 non-asthma subjects, specimens from the 2 asthma subjects showed expression of $CysLT_1$ receptor on the bronchial smooth muscle, bronchial epithelium, and alveolar leukocytes (Fig. 2). Regarding $CysLT_2$ receptors as well, specimens from both 2 asthma subjects showed the expression on the bronchial smooth muscle, bronchial epithelium, and alveolar leukocytes (Fig. 2). In addition, $CysLT_2$ receptor-positive sites were detected in mucus including leukocytes, which may have been released in the bronchial lumen of the 2 asthma subjects.

When asthma lung tissues were stained with rabbit IgG in place of anti-$CysLT_1$ or anti-$CysLT_2$ receptor antibody, no immunostaining sites were detected as shown in Supplementary Figure 1.

Effects of CysLT receptor antagonists on LTD_4-induced intracellular calcium mobilization in $CysLT_1$ or $CysLT_2$ receptor-expressing cells

ONO-6950 inhibited both $CysLT_1$ and $CysLT_2$ receptors-mediated response in LTD_4-induced calcium mobilization assay with IC_{50} values of 1.7 and 25 nM, respectively (Table 2). This inhibition was less than that of montelukast for $CysLT_1$ receptor-mediated response (IC_{50} value of 0.46 nM), but comparable to that reported for Bay$CysLT_2$RA against $CysLT_2$ receptor-mediated response (IC_{50} value of 14 nM).[17] Montelukast showed very weak inhibition of $CysLT_2$ receptor-mediated response (IC_{50} value of 1800 nM), while Bay$CysLT_2$RA inhibition of $CysLT_1$ receptor-mediated response is reported to be even weaker (IC_{50} value of 2500 nM).[17]

Effects of CysLT receptor antagonists on LTD_4- or antigen-induced bronchial smooth muscle contractions

As shown in Figure 3A, ONO-6950 concentration-dependently inhibited LTD_4-induced contractions in the bronchi isolated from non-asthma subjects with almost complete inhibition at 1000 nM (Fig. 3A). This effect was comparable to that of montelukast (100 nM), suggesting that ONO-6950 antagonistic activity for the $CysLT_1$ receptor is approximately 10-fold less than that of montelukast. As for Bay$CysLT_2$RA, this $CysLT_2$ receptor antagonist at

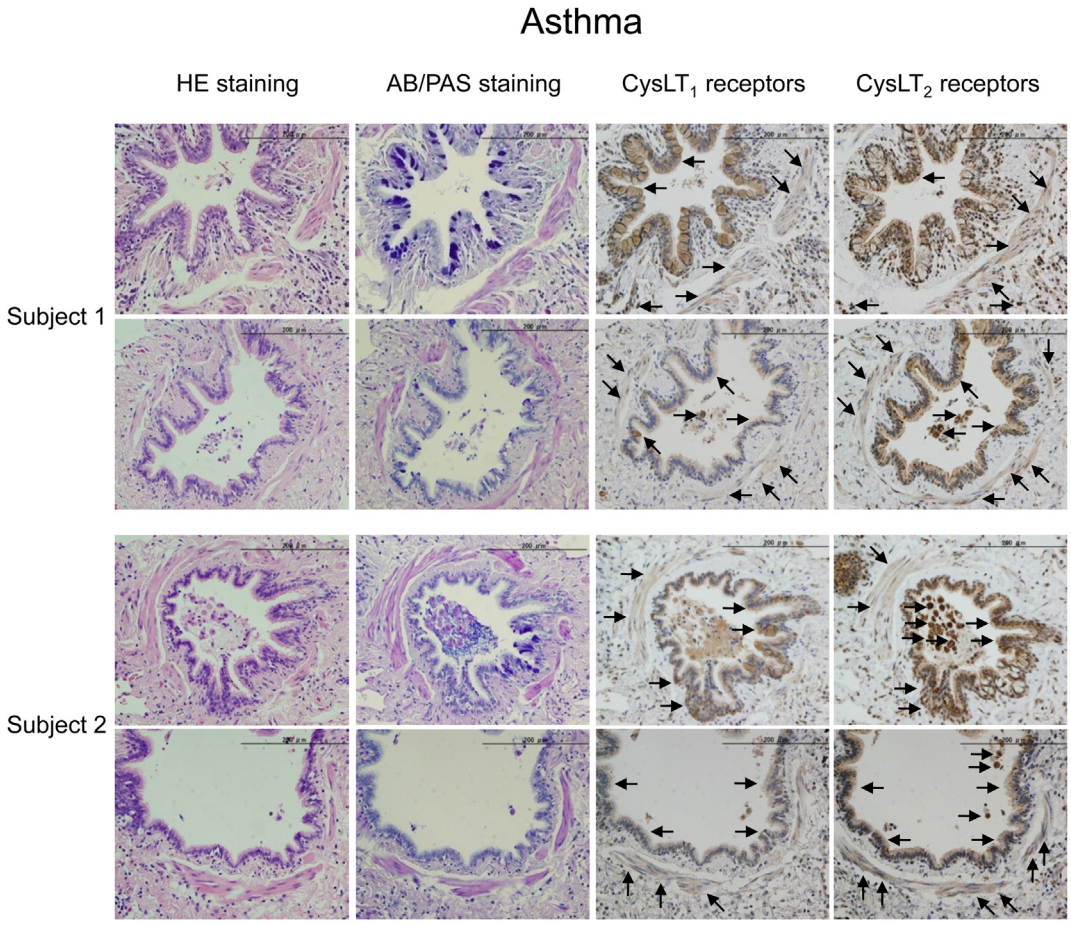

Fig. 2. Immunohistochemical micrographs of lung tissues from the 2 asthma subjects. The tissues were cut into 4 serial sections (4-μm thick). Two of the sections were stained with hematoxylin and eosin (HE), and then with alcian blue/periodic acid-Schiff (AB/PAS). The other two sections were used in immunohistochemical determination of $CysLT_1$ and $CysLT_2$ receptors expression. Arrows show representative immunoreactive sites for each receptor.

Table 2
ONO-6950 and montelukast IC_{50} values for inhibition of LTD_4-induced intracellular calcium mobilization response in human $CysLT_1$ receptor- or human $CysLT_2$ receptor-expressing cells.

	IC_{50} value (nM)	
	$CysLT_1$ receptor	$CysLT_2$ receptor
ONO-6950	1.7 (1.2–2.4)	25 (19–32)
Montelukast	0.46 (0.37–0.58)	1800 (1500–2100)

Fura 2-AM-loaded CHO-K1 cells expressing human $CysLT_1$ or human $CysLT_2$ receptor were stimulated with LTD_4 at 100 nM, for $CysLT_1$ receptors, or LTD_4 at 0.3 nM, for $CysLT_2$ receptors. IC_{50} values and 95% confidence intervals shown in parentheses were estimated from a 2-parameter logistic model using the results of 5 experiments. The cells, treated with CysLT receptor antagonists, were incubated for 30 min before LTD_4 addition.

300 nM had no effect on LTD_4-induced contractions in the bronchi isolated from the non-asthma subjects (Fig. 3B).

Figure 4 represents effects on antigen-induced contraction of bronchi isolated from non-asthma subjects. Both ONO-6950 (300 nM) and montelukast (30 nM) clearly, but only partially, inhibited antigen-induced bronchial smooth muscle contractions, especially 30–90 min after antigen challenge (Fig. 4). $BayCysLT_2RA$ (100 nM), on the other hand, had no inhibitory or potentiating effect on such contractions, even at the high concentration of 1000 nM (data not shown). When the $CysLT_2$ receptor-specific antagonist $BayCysLT_2RA$ was used in combination with montelukast, it neither potentiated nor weakened montelukast-induced inhibition of antigen-induced bronchial smooth muscle contractions (Fig. 4).

In the bronchi isolated from the asthma subject 1, both the $CysLT_1$-specific antagonist montelukast and the $CysLT_2$-specific antagonist $BayCysLT_2RA$, partially inhibited antigen-induced airway smooth muscle contractions (Fig. 5A, Supplementary Fig. 2A). Interestingly, combination of montelukast and $BayCysLT_2RA$ produced additive strong inhibition of such contractions (Fig. 5A, Supplementary Fig. 2A). Consistent with this finding, the dual $CysLT_{1/2}$ antagonist ONO-6950 inhibited antigen-induced airway smooth muscle contractions with a potency equivalent to that of montelukast and $BayCysLT_2RA$ combination treatment (Fig. 5A, Supplementary Fig. 2A). It is noteworthy to mention here that the baseline of montelukast-treated bronchus was relatively high at antigen challenge, probably due to unstable baseline during pre-treatment with montelukast, pyrilamine and indomethacin (Fig. 5A, Supplementary Fig. 2A).

As for the bronchi isolated from the asthma subject 2, only preparations treated with antagonists of the $CysLT_1$ receptor (montelukast or ONO-6950) showed reduced contractions (Fig. 5B, Supplementary Fig. 2B).

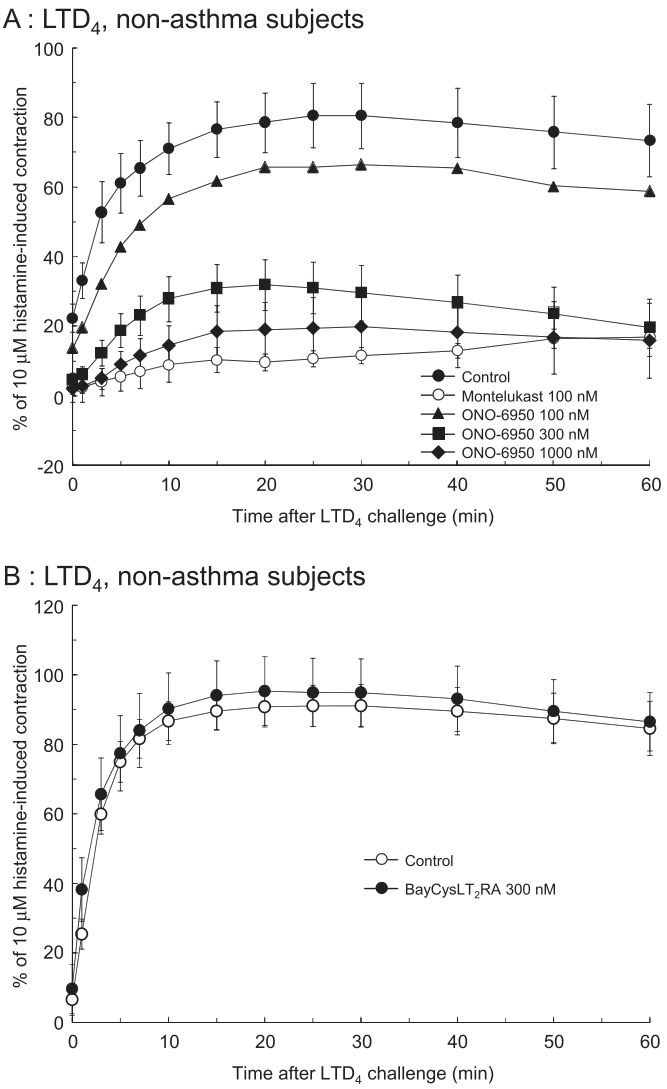

Fig. 3. (**A**) Effects of ONO-6950 and montelukast on LTD_4-induced contractions in bronchi isolated from non-asthma subjects. ONO-6950 (100, 300 and 1000 nM), montelukast (100 nM) or vehicle (0.1% DMSO) was added 30 min before addition of LTD_4 (30 nM). Each point represents the mean or mean ± S.E. of 2–4 experiments. (**B**) Effect of $BayCysLT_2RA$ on LTD_4-induced contractions in bronchi isolated from non-asthma subjects. $BayCysLT_2RA$ (300 nM) or vehicle (0.1% DMSO) was added 30 min before addition of LTD_4 (30 nM). Each point represents the mean ± S.E. of 8 experiments.

Discussion

In the present study, we showed that $CysLT_1$ receptors were expressed on the bronchial smooth muscle and epithelium, and on alveolar leukocytes in all non-asthma and asthma subjects. On the other hand, $CysLT_2$ receptors were expressed on these airway cells in 2 in 2 asthma subjects, whereas they were detected in only a part of non-asthma subjects. Furthermore, our results show that blockage of $CysLT_2$ receptors suppressed antigen-induced bronchial smooth muscle contractions in lung tissue preparations from one of the two asthma subjects.

One of the limitations of our study is that we were only able to use 2 asthma samples. It is quite difficult to obtain sufficient lung tissues from asthma patients for research. However, it should be noted that we used serial tissue sections that allowed co-expression of $CysLT_2$ and $CysLT_1$ receptors at the same sites. To our knowledge, this is the first report demonstrating the concurrent expression of $CysLT_2$ receptors with $CysLT_1$ receptors in airway tissues of actual asthma subjects compared to non-asthma subjects. In addition, our findings suggest that $CysLT_2$ receptors are functionally involved in asthma bronchoconstriction.

It is also interesting to note that the asthma subjects recruited in this study had mild symptoms compared to those of the so-called “severe asthma”, since asthma subject 1 and 2 were classified as “mild persistent” and “mild intermittent”, and were treated with a medium dose ICS (treatment step 2) and a low dose ICS (treatment step 1), respectively. It may therefore be speculated that expression of $CysLT_2$ receptors is further up-regulated in subjects with severe asthma or asthma exacerbation. Indeed, it is reported that asthma exacerbation triggers $CysLT_2$ receptors expression in eosinophils, a phenomenon not observed during stable periods.[19] On the other hand, Negri et al.[20] have demonstrated that fluticasone inhibited IL-

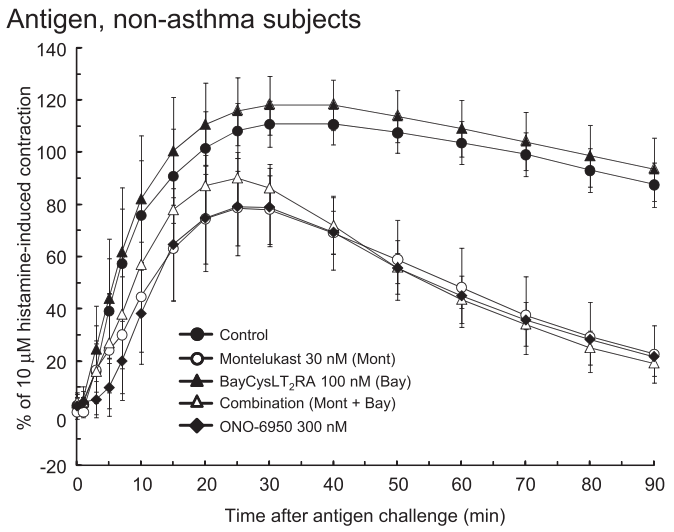

Fig. 4. Effects of ONO-6950, montelukast, BayCysLT$_2$RA, and combination of montelukast and BayCysLT$_2$RA on mite antigen-induced contractions in bronchi isolated from the non-asthma subjects. The bronchi were passively sensitized with atopic serum before being suspended in Magnus bath. ONO-6950 (300 nM), montelukast (30 nM), BayCysLT$_2$RA (100 nM) or vehicle (0.1% DMSO) was added 30 min before antigen challenge. A cyclooxygenase inhibitor, indomethacin and an anti-histamine drug, pyrilamine were applied 10 and 5 min before antigen challenge, respectively, to make antigen-induced contractile response as much as possible dependent on CysLTs activity. Each point represents the mean ± S.E. of 4 or 5 experiments.

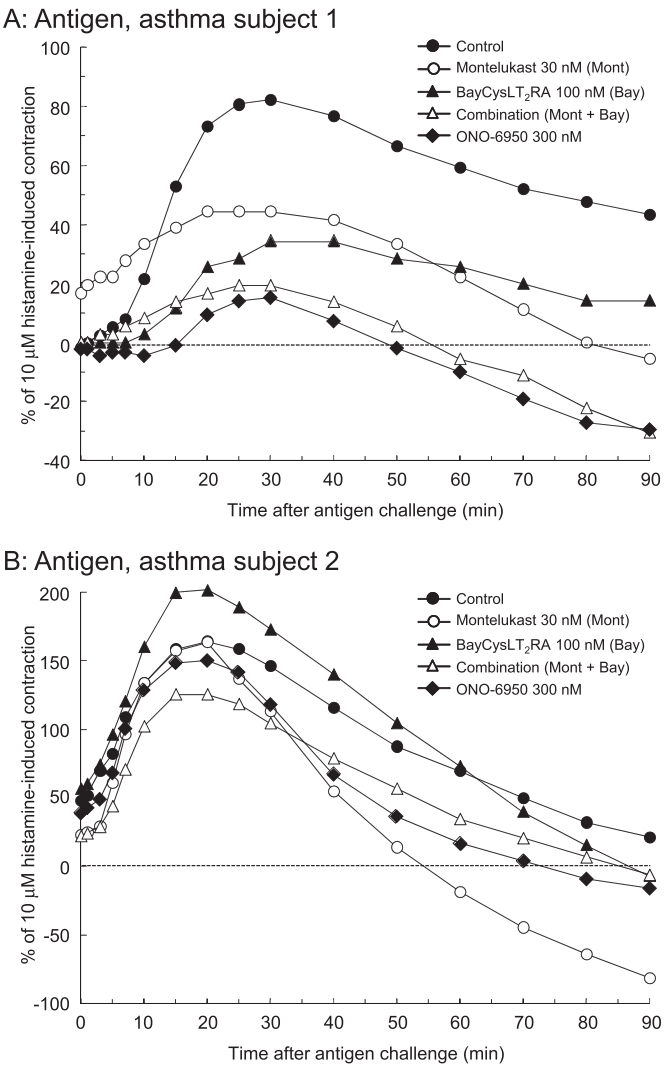

Fig. 5. Effects of ONO-6950, montelukast, BayCysLT$_2$RA, and combination of montelukast and BayCysLT$_2$RA on mite antigen-induced contractions in bronchi isolated from the 2 asthma subjects (**A**: Subject 1; **B**: Subject 2). The experimental procedure is the same as that given in the legend of Fig. 4. Each point represents the value obtained in each experiment.

4-induced CysLT$_2$ receptors protein expression, but not CysLT$_1$ receptors, on monocytes, T cells, and eosinophils. Because the asthma subjects were treated with ICS/LABA, the CysLT$_2$ receptors expression may had been negatively regulated. Regarding effect of LABA on CysLT receptors expression, there has been no study to our knowledge.

In agreement with our results showing the presence of CysLT$_2$ receptors in alveolar leukocytes, both CysLT$_1$ and CysLT$_2$ receptors have been reported to be expressed not only in macrophages,[9] but also in eosinophils,[19,21] mast cells,[22] basophils,[23] and dendritic cells.[24] The functional importance of CysLT$_2$ receptors in these leukocytes was not addressed in this study. However, Jiang et al.[22] have shown that both CysLT$_1$ and CysLT$_2$ receptors are expressed on the membranes and nuclei of a human mast cell line, and that knockdown of CysLT$_2$ receptors increases CysLT$_1$ receptors surface expression. These findings suggest that activation of CysLT$_2$ receptors down-regulates CysLT$_1$ receptors expression.

In addition to CysLT$_2$ receptors activation negative feedback on CysLT$_1$ receptors expression, a broader functional regulation between CysLT$_1$ and CysLT$_2$ receptors has also been reported: Knockdown of CysLT$_2$ receptors was reported to increase CysLT$_1$ receptor-dependent proliferation of human mast cells.[22] Barrett et al.[24] have shown that *D. farinae* sensitization and challenge in CysLT$_2$ receptor-deficient mice results in a marked increase in eosinophilic pulmonary inflammation, serum IgE level, and Th2 cytokine level. Maekawa et al.[25] have also reported that leukotriene-induced ear edema shows a delayed peak response in CysLT$_2$ receptor-deficient mice compared to wild-type mice. These findings suggest that activation of CysLT$_2$ receptors down-regulates not only CysLT$_1$ receptors expression, but also CysLT$_1$ receptor-mediated biological and inflammatory responses. However, as shown in this study, treatment with the CysLT$_2$ receptor antagonist BayCysLT$_2$RA did not reverse montelukast-induced inhibition of anaphylactic bronchoconstriction in specimens from both the non-asthma and asthma subjects. Therefore, CysLT$_1$ receptors activation may be differently regulated by CysLT$_2$ receptors expression, at least in human bronchial contractions. Alternatively, the observed discrepancy may be due to species difference.

It is now widely known that blockade of CysLT$_1$ receptors strongly inhibits antigen-induced bronchial contractions in specimens isolated from non-asthma subjects. However, this blockade is not complete as shown in the present study and in other literature.[4,15,16] The results of the current study suggest that CysLT$_2$ receptors activation has no significant role in the bronchial contractions recorded in the non-asthma specimens. On the other hand, in one of the two asthma specimens, CysLT$_2$ receptors blockade inhibited anaphylactic bronchoconstriction. This inhibition was potentiated by dual blockade of CysLT$_1$ and CysLT$_2$ receptors. These results suggest that there may be a certain asthma background, in which activation of CysLT$_2$ receptors is involved in anaphylactic bronchocontractile response, and thus may play a significant role in asthma response in certain asthma population.

It is not clear why the involvement of CysLT$_2$ receptor activation in anaphylactic response was different between the 2 asthma specimens, even though CysLT$_2$ receptors were expressed in both specimens to a similar degree. Mechanisms other than increased

expression of $CysLT_2$ receptors, such as functional up-regulation, may also be involved in this response. It is intriguing to speculate that asthma background affects such functional up-regulation. On the other hand, it should be noted that one lung sample showing $CysLT_2$ receptors contribution to bronchoconstriction was derived from an asthma subject (asthma subject 1), who had had a history of atopy, whereas the other subject (asthma subject 2) had not. In addition, asthma subject 1 had a relatively high percentage of eosinophils in peripheral blood leukocytes (6.8%). Moreover, asthma subject 1 was non-smoker, whereas asthma subject 2 was smoker. It is therefore suggested that these subjects backgrounds may have affected the function of $CysLT_2$ receptors. However, further studies are needed to clarify the exact role of $CysLT_2$ receptors in asthma pathogenesis.

Henderson et al.[26] have demonstrated that montelukast blockade of $CysLT_1$ receptors improves airway remodeling, including airway goblet cell metaplasia, smooth muscle cell layer thickening, and subepithelial fibrosis in a mouse model of asthma. The histological findings of this study revealed a thickened bronchial epithelium exhibiting epithelial cells filled with AB/PAS-positive mucus in the specimens prepared from the asthma patients. Interestingly, in these specimens, $CysLT_1$ receptors tended to be expressed on the mucus-positive epithelium, suggesting that $CysLT_1$ receptors activation is involved in the development of epithelial remodeling in humans as reported in mice.[26] It has also been reported that CysLTs mediate Th2 cell-dependent pulmonary inflammation through activation of $CysLT_1$ receptors in mice.[27,28] Considering the fact that both $CysLT_2$ and $CysLT_1$ receptors are highly expressed in the airway epithelium, it is possible that $CysLT_2$ receptors also play a role in the development of airway remodeling. This hypothesis need to be further investigated both *in vitro* and *in vivo*.

In conclusion, we have shown in this study that $CysLT_2$ receptors were expressed in lung specimens isolated from 2 asthma subjects. This $CysLT_2$ receptors expression may contribute to antigen-induced bronchoconstriction in certain asthma cases. These results imply that $CysLT_2$ receptor antagonists, including BayCysLT$_2$RA and ONO-6950, may be useful for the treatment of the certain asthma population. However, because the present findings were led from only 2 asthma specimens, further preclinical and clinical studies on $CysLT_2$-or $CysLT_{1/2}$-receptor antagonists are required.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.alit.2015.04.008.

Conflict of interest

TS, MK and KK are employees of Ono Pharmaceutical Co., Ltd, Osaka, Japan. TN received a research grant from Ono Pharmaceutical Co., Ltd. The rest of the authors have no conflict of interest.

Authors' contributions

TA and MH were involved in the recruitment of volunteers and in data interpretation. SK was involved in the design of the study and in data interpretation. KK was involved in the study design, data interpretation and manuscript preparation. MF contributed to data collection, analysis and interpretation. TS, MK and TN were involved in the study design, data collection, data interpretation, data analysis and writing the manuscript. TS and TN have full access to the data, and are responsible for the integrity of data and final decision to submit this manuscript. All authors have approved the final version of this manuscript for submission.

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